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Polymerization of a Quinone-crosslinked Marine Bioadhesive

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Studies conducted over the past year on DOPA-containing proteins from G. demissa and M. edulis suggest that these form soluble, stable complexes with type II collagen, essentially shielding the latter from digestion by clostridial collagenase. No such shielding was conferred by the Fasciola DOPA-proteins. Catecholoxidase-catalyzed oxidation of the DOPA residues to o-quinones results in a substantial tautomerization of the quinones to α,ß-dehydro DOPA. The rate of tautomerization at physiological pH is comparable to the proposed Michael addition of lysyl epsilon amino groups to quinones. This may suggest that α,ß-dehydro DOPA is an important intermediate in quinone-tanning. A new histidine-rich DOPA-containing egg shell precursor has been characterized from Fasciola hepatica.

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DATE: 15 October 1989

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PRINCIPAL INVESTIGATOR: Herbert Waite

CONTRACTOR: University of Delaware - College of Marine Studies

CONTRACT TITLE: Polymerization of a Quinone-Tanned Marine Bioadhesive Protein

START DATE: October 1986

RESEARCH OBJECTIVES: This research has had two main objectives: 1) to survey the biochemical diversity of DOPA-containing marine adhesive proteins and cements, and 2) to examine the chemical mechanism of quinone-tanning *i.e.* cross-linking of DOPA-containing proteins.

Summary of Plans for the Next Year of Support:

Research on marine adhesives will continue with the proposal entitled "Marine Cement: Anatomy of a Natural Composite Material". This proposal has the following goals:

A. to isolate and characterize the cross-linking catalyst (catecholoxidase) from mussel cement and determine how the expression of this enzyme is regulated during mussel post-larval development.

B. to isolate and characterize byssal collagen which functions as a fibrous filler in the mussel cement.

C. to analyze the molecular interactions between the DOPA-containing proteins and collagen using bifunctional cross-linking agents.

Summary of Recent Research Progress

Glue Protein from Ribbed Mussels: One of the goals of the past year has been the characterization and properties of the adhesive protein of ribbed mussels, *Geukensia demissa.* Like their mytilid cousins, ribbed mussels attach themselves to solid objects in estuaries by spinning adhesive byssal threads tipped with adhesive plaques. The adhesive precursor of the plaques was isolated in

quantity from the foot of the mussel. The protein has a apparent molecular weight of 130,000, a pI of 8.1, and contains a high proportion of Gly, Gln/Glu, Lys and 3,4-dihydroxyphenylalanine (DOPA). The sequence of various tryptic peptides derived from this protein suggests a pattern of repeated motifs, such as

Gly-DOPA-Lys, and
X-Gly-DOPA-Y-Z-Gly-DOPA-/Tyr-Lys

where X is Thr or Ala in octapeptides and Gln-Thr in nonapeptides. Y is variable, but, more often than not, hydrophobic, and Z is frerquently Pro or 4-*trans*-hydroxyproline. The presence of Pro-Gly and Hyp-Gly sequences, and the presence of δ-hydroxylysine in the protein is reminiscent of collagens; however, the protein is not labile to clostridial collagenase, nor does collagen cross-react with antibodies raised against the mussel protein. Unlike typical collagens, Gly probably occurs only at every 4th or 5th residue in this unusual protein. This protein brings to six the number of sequenced DOPA-containing preqursors of quinone-tanning.

Specific Interaction between Collagen and Mussel Glue Protein: Perhaps related to the iso-electric point of the glue protein from ribbed mussels is its limited solubility at physiological pH. At a concentration of 1 mg/ ml in 0.1 M phosphate, Tris, or Hepes at pH 8.0, it takes only a few minutes for the protein to precipitate out of solution. In 0.1 M borate at pH 8.0, a soluble DOPA-borate complex is formed by the protein. This, however, undergoes rapid adsorption to the walls of the reaction vessel. Adsorption is measured as the amount of protein remaining in solution as indicated by the intensity of staining of aliquots by Coomassie Blue R-250 following acid-urea polyacrylamide gel electrophoresis or by amino acid analysis following hydrolysis. Reaction conditions consist of 1 mL of 0.05-0.1 M borate at pH 8.0 containing 0.5 mg/ mL under constant stirring at 22 °C. In an attempt to determine whether this adsorption is competitively reversible, we added another surface, *i.e.* type II collagen from calf cartilage, to the mixture. The final concentration of added collagen was 0.5 mg/mL. Periodic aliquots of the glue protein/collagen mixture run on gels suggest that the concentrations of both remain essentially invariant over the course of the experiment. In an attempt to determine if the glue protein remains in solution by adsorbing preferentially to collagen, we added clostridial collagenase (50 μg/mL). This enzyme specifically digests collagen and gelatin but not glue protein. When glue protein is present, the collagenase is unable to digest the collagen even after 6 h of exposure. The shielding effect is evident at glue protein to collagen ratios as low as 1:100 before collagen degradation becomes apparent in the observed time interval. Is the glue protein forming a stable complex with the native collagen so as to shield it from digestion, or is it simply inhibiting the collagenase directly?

We postulated that since the glue protein contains DOPA, a functional group with considerable affinity for metals (Pierpont and Buchanan, 1981), and the ezyme is a metalloenzyme with Zn at the active site (Seifter & Harper, 1971), the DOPA might be chelating Zn from the collagenase. This scenario, however, is not likely in view of the susceptibility of heat denatured collagen to digestion. Moreover, if Zn chelation plays any role at all in the shielding effect, it should be possible to titrate the DOPA residues with $ZnSO_4$, for example, and observe an induction of enzyme activity as the DOPA-Zn binding capacity is saturated. Addition of Zn in molar proportions of 1:1, 1:10. 1:100, and 1:1000 to glue protein did not lead to any apparent degradation of the native collagen. Moreover, the digestion of collagen by collagenase is undeterred by vitelline protein B, a DOPA-containing protein from *Fasciola hepatica* (Waite & Rice-Ficht, 1987). These results suggest that simple binding of prosthetic Zn in the collagenase by catechols or peptidylDOPA is not a likely mode of action. A more specific and energetic interaction between the two molecules is indicated.

Catecholoxidase from Ribbed Mussel Byssus. Byssal catecholoxidase is a remarkable enzyme in at least two respects: 1) it catalyzes the polymerization (or quinone-tanning) of the DOPA- containing glue protein and in this way retards the decomposition of the byssus, and 2) in the byssal thread cortex, the enzyme is present at concentrations of about 50 mol% with the balance due to the glue protein. At these concentrations, the enzyme is unlikely to be a classical Michaelis-Menten catalyst, but rather something more akin to a solid state catalyst with catalytic as well as structural functions. The purified enzyme has an apparent molecular of 38,000, a specific activity of quinone units per mg protein, a blocked N-terminus, and an amino acid composition in which 4 residues account for 60 mol % of all those in the protein, *ie.* Ser, Glu, Asp, Gly. We are attempting to raise antibodies to this enzyme to be used to screen a mussel expression library

The Glue Proteins from Liver Flukes: The vitellaria are an extensive network of glandular cells and ducts distributed throughout the peripheral tissues of the liver fluke *Fasciola hepatica.* Eggshell precursor proteins are produced and stockpiled in the vitelline cells of mature flukes. Vitelline protein C has an extraordinary composition: the amino acid 3,4-dihydroxyphenyl-L-alanine (DOPA) and histidine each comprise about 20 % of the residues while glycine represents 41-42 % in all variants of what appears to be a microheterogeneous protein family. Protein C has an apparent molecular weight of 16-17 000 by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. Although the protein appears homogeneous following polyacrylamide gel electrophoresis in Tris-glycine with SDS and in acetic acid-urea, electrophoresis in borate, however, suggests that the vitelline protein consists of 4 or more closely related proteins weighing

from 16-18 500. Isoelectric focussing of the protein family in the presence of 8 M urea resolves only two species having *pI* values of 6.89 and 6.99. A single N-terminus having the sequence H-H-W-D-G-DOPA-G-DOPA-G was detected. The primary structure of vitelline protein C is characterized by a repeated motif consisting of $(G-X)_n$ where X is Ser, DOPA, or His. Most of the His occurs as G-H repeats in a pepsin-resistant fragment of the protein. Previously, a 31 kDa protein, representing up to 6 % of the total protein in the fluke, was reported (Waite, J.H., and Rice-Ficht, A. (1987) *Biochemistry 26*, 7819-7825) to contain significant levels of DOPA. In other respects, however, it is distinct from vitelline protein C. Present studies suggest the existence of at least one other distinct DOPA-containing protein in the vitellaria.

Enzyme Oxidation of Glue Proteins: Proteins containing the post-translationally modified amino acid DOPA undergo natural auto-sclerotization as an integral part of their bioadhesive or structural function. In last year's summary, we reported that addition of catalytic amounts of mushroom polyphenoloxidase to solutions of mussel glue protein increased the rate of glue protein adsorption to glass and polyproylene surfaces by an order of magnitude. To clarify the chemical mechanism of sclerotization, we examined the oxidation products of simple analogues of the peptidyl DOPA moiety, such as N-acetyl-L-DOPA-methyl ester (I in Fig. 1) together with a number of small peptides. Oxidation was effected by two catechoxidases and by sodium metaperiodate. The catecholoxidases were a) commercial mushroom polyphenoloxidase (*Sigma*), and b) soluble enzyme extracted from the byssal threads of *Geukensia demissa* (Rzepecki et al., 1989). In the course of this research, we developed a sensitive colorimetric assay of enzyme activity which is based on the addition of L-proline to *o*-quinone, the latter being formed from *o*-diphenols by catecholoxidase-catalysed oxidation. The N-prolyl-*o*-quinone has a molar extinction coefficient of 8000-9000 cm^{-1} at 390 nm (Rzepecki & Waite, 1989). The reaction products were characterized spectroscopically using the borate difference method of Waite (1984) and by HPLC and amino acid analysis. Following enzymic or nonenzymic oxidation of DOPA analogues to the corresponding quinones, a spontaneous rearrangement of the quinone to an α,β-dehydroDOPA moiety (IV) occurred (Fig. 1). The dehydroDOPA is very likely in resonance with the enamine in which the double bond shifts to the amide nitrogen. Similar oxidative rearrangement occurred with 3,4-dihydroxyhydrocinnamic acid, a non-aminated DOPA analogue, but not with N-acetyldopamine. A variety of peptides, including Ala-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys, a synthetic analogue of the consensus decapeptide in the glue protein of *Mytilus edulis*, show spectral characteristics consistent with the same rearrangement. The existence of this novel rearrangement of Dopa residues has important implications for the conformations of such proteins and for the types of covalent cross-links involved in catecholoxidase-catalyzed auto-sclerotization. Dehydroamino

acids influence protein conformation, possibly by stabilizing β-turn structures (Imazu et al., 1988). In this regard, it is intriguing that Rudall (1955) predicted that *Mytilus* byssal threads consist of a stack of packed β-sheets. The mere formation of α,β-dehydroDOPA groups in the protein might serve to enhance the stability of the β-sheets.

Recent kinetic studies of the oxidation of peptidyl-DOPA analogues by periodate or catecholoxidases in the pH range 6.0 to 7.2 show that only two chemical species absorbing significantly between 320 and 430 nm are formed, one of which was the quinone (II in Figure 1) formed immediately, and the other, with a λ_{max} at 322 nm, corresponded to α,β-dehydroDOPA (IV). Calculation of the extinction coefficients for the quinone and dehydroDOPA facilitates the estimation of the rates k'_q and k'_p corresponding to quinone decay and dehydroDOPA formation, respectively (Table I). At pH 6.0, log transformation of the data showed that the reaction proceeded by a 1st order mechanism, and that conversion of the DOPA-quinone to dehydroDOPA was stoichiometric, that is, all quinone degradation was accounted for by dehydroDOPA. At higher pH values, conversions were increasingly less efficient (30-40 %) since the quinone (II) formed a variety of other products (Figure 1). The mechanism of dehydroDOPA formation is probably significant in assessing the putative function of dehydroDOPA in DOPA-containing proteins, hence it was interesting that when peptidylDOPA was oxidized by amounts of periodate in excess of eqimolarity, there was a lag phase before the onset of quinone decay or dehydroDOPA formation. This lag phase increased with increasing amounts of periodate added and lasted about 20-30 min at a 2-fold excess of periodate over peptidylDOPA. Addition of 4-methylcatechol during the lag phase resulted in immediate dehydroDOPA formation. Thus DOPA-quinone is unable to undergo rearrangement to dehydroDOPA spontaneously and requires an *o*-diphenol such as DOPA as a cofactor. A model for the possible reactions is presented in Fig. 1. Peptidyl DOPA is oxidized in reaction 1 to its quinone (II) which may then react with excess DOPA to form a charge transfer complex (X) (reaction 2). This complex can breakdown to form the original compounds or may result in a quinone methide (III) which undergoes rapid rearrangement to dehydroDOPA (IV). The rate constant for dehydroDOPA formation (k'_p) is really pseudo-first order since the catechol would be regenerated after reaction 2. These results suggest that in DOPA proteins, dehydroDOPA formation would require the participation of neighboring DOPA residues following the oxidation of one of these residues. Provided sufficient flexibility were present in the protein, even a few remaining unoxidized DOPA residues might serve catalytically to produce dehydroDOPA.

Conclusions :

A. Ribbed mussels have a glue protein with a primary sequence that is significantly different from that of *Mytilus.*

B. Ribbed mussel glue protein preferentially adsorbs to and shields type II collagen from the action of clostridial collagenase.

C. A unique eggshell precursor protein has been isolated from liver flukes. It contains 40% glycine, 20% histidine and 20% DOPA and presumably serves as a natural varnish.

D. Catecholoxidase-formed peptidyl-quinones spontaneously rearrange to form peptidyl-dehydroDOPA. The rearrangement reaction appears to require unmodified peptidyl-DOPA as a primer with reducing activity.

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Training Activities: At present, *two* graduate students (Stefan Samulewicz & Kathleen Little) and *one* post-doctoral fellow (Lesz Rzepecki) are being supported by this contract. In December, they will be joined by another post-doctoral fellow (Karolyn Mueller) who will have completed the ONR-sponsored course in marine molecular biology on Catalina Island.

Women or minorities	- 2
Non-citizens	- 1

Figure 1. Hypothetical reaction pathways of peptidyl-DOPA oxidation.

Fig. 1

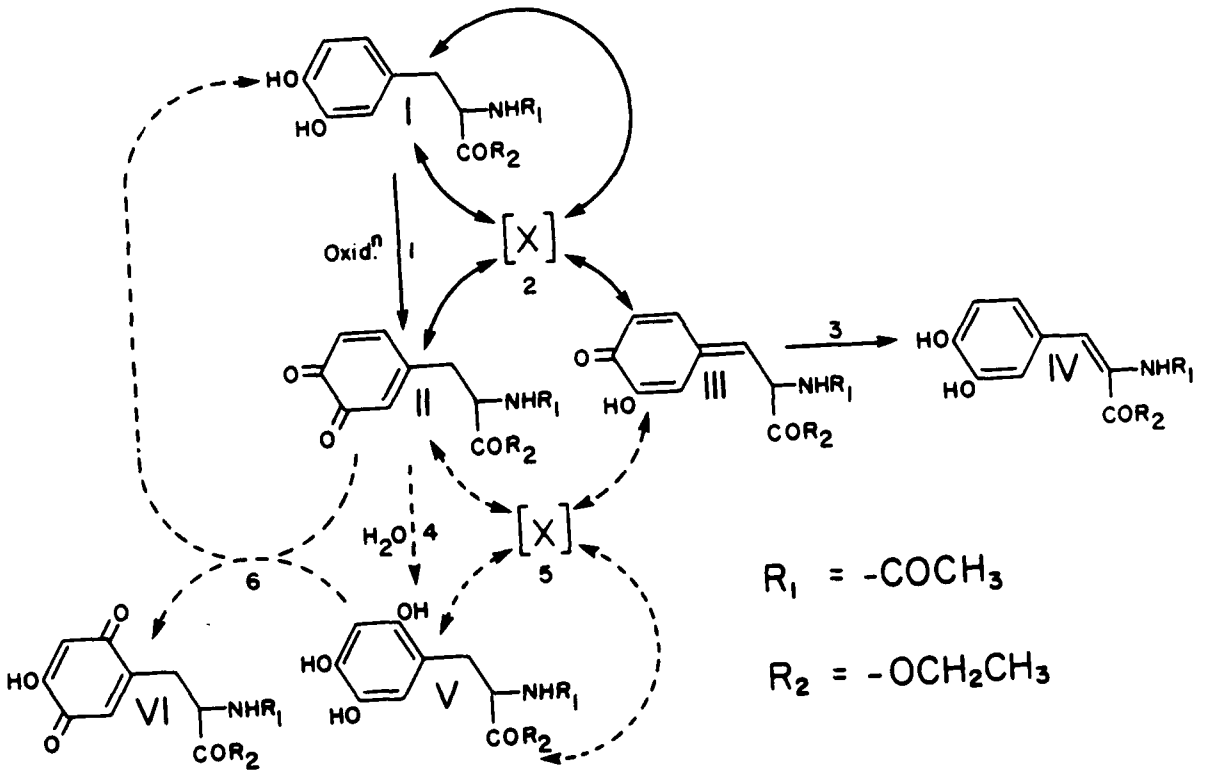
HO
HO
I
NHR1
COR2
Oxidn
1
[X]
2
II
III
3
IV
H2O
4
[X]
5
6
VI
V
OH
R1 = -COCH3
R2 = -OCH2CH3

Table I

pH	initial [quinone] μM	(a) $k'_q \times 10^4$	(a) $k'_p \times 10^4$	(a) k'_p/k'_q	(b) approximate range of $\Delta p/\Delta p$
6.0	34.7	9.19 (8.76)	8.89 (8.59)	0.97 (0.98)	1.03 - 1.04
	52.2	8.28 (7.94)	7.97 (8.20)	0.96 (1.03)	
	68.3	10.73 (7.62)	8.45 (8.08)	0.78 (1.06)	
	99.1	5.57 (6.22)	6.41 (7.65)	1.15 (1.24)	
7.2	32.6	43.3	25.8	0.60	0.65 - 0.52
	49.6	35.3	23.2	0.66	
	68.0	30.7	20.0	0.65	
	101.1	17.6	15.3	0.87	
8.0	29.5	74.6	29.5	0.40	0.40 - 0.30
	46.3	57.7	22.5	0.39	
	65.1	52.4	23.4	0.45	

(a) values of k'_q and k'_p obtained from initial rates (measured over the first 20 s of reaction). Values in brackets obtained from logarithmic transformation of progress curves at pH 6.0 only.
(b) The range of $\Delta p/\Delta q$ was obtained from Δp and Δq measured for the intervals 20-60s and 220-260 s of the corrected rate curves, and is reported as the mean values for all initial quinone concentrations used. The symbols p and q represent NAcΔDEE* and NAcDEE** quinone concentration respectively.

* Dehydro DOPA

** peptidyl DOPA quinone

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